A Photoisomerizable Muscarinic Antagonist

Studies of Binding and of Conductance Relaxations in Frog Heart

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ABSTRACT These experiments employ the photoisomerizable compound, 3,3′-*bis*-[α-(trimethylammonium)methyl]azobenzene (Bis-Q), to study the response to muscarinic agents in frog myocardium. In homogenates from the heart, *trans*-Bis-Q blocks the binding of [^{3}H]-*N*-methylscopolamine to muscarinic receptors. In voltage-clamped atrial trabeculae, *trans*-Bis-Q blocks the agonist-induced potassium conductance. The equilibrium dose-response curve for carbachol is shifted to the right, suggesting competitive blockade. Both the biochemical and electrophysiological data yield a dissociation constant of 4–5 μM for *trans*-Bis-Q; the *cis* configuration is severalfold less potent as a muscarinic blocker. Voltage-clamped preparations were exposed simultaneously to carbachol and Bis-Q and were subjected to appropriately filtered flashes (<1 ms duration) from a xenon flashlamp. *Trans* → *cis* and *cis* → *trans* photoisomerizations cause small (<20%) increases and decreases, respectively, in the agonist-induced current. The relaxation follows an S-shaped time course, including an initial delay or period of zero slope. The entire waveform is described by $[1 - \exp(-kt)]^n$. At 23°C, k is ~3 s^{-1} and n is 2. Neither k nor n is affected when: (*a*) [Bis-Q] is varied between 5 and 100 μM; (*b*) [carbachol] is varied between 1 and 50 μM; (*c*) carbachol is replaced by other agonists (muscarine, acetylcholine, or acetyl-β-methylcholine); or (*d*) the voltage is varied between the normal resting potential and a depolarization of 80 mV. However, in the range of 13–30°C, k increases with temperature; the Q_{10} is between 2 and 2.5. In the same range, n does not change significantly. Like other investigators, we conclude that the activation kinetics of the muscarinic K^+ conductance are not determined by ligand-receptor binding, but rather by a subsequent sequence of two (or more) steps with a high activation energy.

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INTRODUCTION

The effects of acetylcholine on biological membranes encompass a wide range of time scales. The fastest actions occur at the nicotinic synapses of vertebrate skeletal muscle fibers and electroplaques, where acetylcholine receptors control ion channels. The opening and closing rates depend on various factors but are typically on the order of 1 ms^{-1}. Furthermore, the first ion channels open within 10 μs after agonist molecules appear near the receptors (Krouse et al., 1980; Lester et al., 1980*b*).

At other cholinergic synapses the responses are several orders of magnitude slower (reviewed by Bolton, 1979; Koketsu, 1978; Kehoe and Marty, 1980). The best-studied examples of such responses are those of muscarinic acetylcholine receptors, which increase potassium conductance in the heart (Trautwein and Dudel, 1958; Hutter, 1961; Hartzell et al., 1977; Garnier et al., 1978*a*) and decrease it in autonomic neurones (Brown and Adams, 1980); responses with similar characteristics have been noted in *Aplysia* neurones (Kehoe, 1972*a* and *b*; Marty and Ascher, 1978). At such synapses, not only are the responses slow (several hundred milliseconds) but they also start after a delay, or period of zero slope, lasting several tens of milliseconds (del Castillo and Katz, 1955; Purves, 1976; Hartzell et al., 1977).

What is the nature of the molecular events that limit the rate of these muscarinic responses? Such questions can be addressed with experiments that exploit spontaneous or forced perturbations from equilibrium activation. By using photosensitive nicotinic drugs, for instance, it is possible to monitor the membrane conductance while manipulating (*a*) the concentration of drugs near receptors or (*b*) the structure of the drug-receptor complex (Bartels et al., 1971; Lester et al., 1980*a*). A particularly straightforward experiment of this type involves a photochemically induced "concentration jump" of agonist (Lester and Chang, 1977; Krouse et al., 1980).

Unfortunately, no presently known drugs appear to be light-activated muscarinic agonists. We have found, however, that some photoisomerizable azobenzene derivatives bind to muscarinic receptors and block the effect of agonists; these drugs have the additional property that their *cis* and *trans* configurations have different potencies. Thus, the agonist-induced conductance can be regulated with light flashes. We have exploited several advantages of this technique: (*a*) the kinetics of both conductance increases and decreases can be studied; (*b*) these kinetics can be examined for their sensitivity to the nature of the agonist and its concentration, the concentration of antagonist, the membrane voltage, the temperature, and other variables. We find that the kinetics are appreciably sensitive only to temperature, which suggests that the rate-limiting step is not the drug-receptor interaction. The results have been briefly reported elsewhere (Nargeot et al., 1981).

METHODS

Binding Studies

These experiments were carried out at Mill Hill, London, on homogenates of the ventricles of the frog, *Rana temporaria*, as described by Berrie et al. (1979) for the rat

ventricles. Briefly, a 1:100 homogenate in 10 mM NaCl, 10 mM $MgCl_2$, 20 nM Na HEPES, pH 7.0, buffer was preincubated for 10 min at 24°C before addition to a fixed concentration of [^{3}H]-*N*-methylscopolamine (NMS; 55 Ci/nmol, final concentration 3×10^{-10} M) and various concentrations of unlabeled competing ligands. Incubation was carried out for 20 min in the dark at 24°C and was terminated by centrifugation (14,000 *g* for 5 min) as previously described (Hulme et al., 1978). Assays were carried out in quadruplicate and nonspecific binding was defined as the radioactivity bound or trapped in the pellet when the incubation medium contained 10^{-6} M 3-quinuclidinyl-benzilate. The data were analyzed by a nonlinear least-squares procedure.

To produce a solution containing predominantly *cis*-Bis-Q, a solution (10^{-2} M) initially in the *trans* state was irradiated in a silica cuvette using a 366-nm CAMAG UV lamp. In one experiment, the optical density (OD) at 266 nm, the isosbestic point, was unchanged over 3 h, which indicated that there was no photochemical decomposition, whereas the OD 320/266 ratio changed from 4.9 to 1.4. From the OD ratio of 1.4 and the reported ratios for pure *cis*- and *trans*-Bis-Q, we calculated that the irradiated mixture contains 80% *cis*- and 20% *trans*-Bis-Q (Lester et al., 1980*a*). In a second experiment using magnetic stirring during a 4-h irradiation period, an OD ratio of 0.99 was obtained, corresponding to 90% *cis*-Bis-Q.

Electrophysiological Studies

These experiments were performed in Pasadena, Calif., on atrial trabeculae from bullfrogs (*Rana catesbiana*). We have combined two techniques, the sucrose-gap voltage clamp (Rougier et al., 1968) and the flash-induced concentration jump (Lester and Chang, 1977). The composition of the Ringer solution was NaCl, 110.5 mM; KCl, 2.5 mM; $CaCl_2$, 1.8 mM; $MgCl_2$ 2 mM, glucose, 0.5 mM; Tris-HCl, 3 mM (pH 7.8).

ELECTRICAL ARRANGEMENTS The double sucrose-gap voltage-clamp preparation has been described by Rougier et al. (1968) and by Garnier et al. (1978*a*). Voltages are expressed relative to the resting potential in the absence of agonist. The flashes produced fractionally small changes in voltage-clamp currents. To amplify these relaxations without distortion by capacitive coupling, we substracted baselines using a track-and-hold circuit (Nass et al., 1978). Signals were then led to an amplifier and to a filter with a pass band from DC to 50 Hz (24 db/octave roll-off). Data were digitized at a frequency of 100 or 200 Hz and led to a minicomputer that controlled and analyzed the experiments, as previously described (Sheridan and Lester, 1977; Lester, 1978).

OPTICAL ARRANGEMENTS We used the xenon short-arc flashlamp, power supply, and trigger circuit described by Nass et al. (1978) and by Lester et al. (1980*a*). This system's light output rises to a peak in 40 μs and then decays with a time constant of 250 μs. To increase energy in the ultraviolet region, we replaced the previous lens system with quartz components. The condenser (6462; Oriel Corp. of America, Stamford, Conn.) had a diameter and focal length of 35 mm; the secondary focusing lens (6197; Oriel Corp. of America) had a diameter of 35 mm and focal length of 75 mm.

Because of this system's high numerical aperture, there are uncertainties in actinometric measurements of intensity. Table I gives approximate values of photoisomerization potency K_t and K_c determined by absorption measurements on solutions containing Bis-Q (Nass et al., 1978; Lester et al., 1980*a*; R. E. Sheridan and H. A. Lester, unpublished data).

TEMPERATURE CONTROL The Ringer solution was heated or cooled just before it entered the central pool. A thermistor was placed in this pool, near the preparation.

This arrangement probably exaggerated changes from the ambient level, because the two sucrose pools were still perfused with solution at the ambient temperature. We estimate that the error amounted to 20%, and this correction factor has been applied to the measured temperature.

SUBTRACTION OF NONSPECIFIC LIGHT-FLASH RELAXATIONS With the more transparent filters, flashes had transient effects on voltage-clamp currents, even in the absence of photosensitive drugs. These nonspecific relaxations are not eliminated by shielding the electrodes or the sucrose pools from the flash or by blocking the infrared radiation with water or a suitable interference filter. Therefore, the signals probably do not arise from photoelectric effects or temperature jumps. The effects were suppressed completely by blocking the light from the central pool. They may thus arise directly from an effect of light on the myocardial tissue.

With the "UV only" filter, the nonspecific relaxation is a small, transient decrease in current (trace *b* in the top panel of Fig. 5). The relaxation reaches almost full ampitude within 20 ms after the flash—much faster than the signals under study here—and a step of the same size and shape appears on the light-flash relaxations in the presence of both agonist and Bis-Q. It therefore appeared simplest to substract the nonspecific relaxation measured in the control solutions.

With visible flashes, the nonspecific relaxation also reaches nearly full amplitude in a few milliseconds (trace *a* in Fig. 6). However, it differs in two respects from the nonspecific relaxation observed with "UV only" flashes. First, the visible flashes produce a transient *increase* in current. Second, this relaxation is quite small in the absence of agonist—even when large outward currents are flowing through the delayed rectifier channels—and increases in size with the agonist-induced current. This relaxation is small or absent during hyperpolarizations that produce inward agonist-induced current, but this is not surprising because the inward currents are themselves small (cf. Garnier et al., 1978*a*; Noma and Trautwein, 1978). Comparable nonspecific effects were observed with all agonists studied. To substract this nonspecific effect, we exploited the fact that nonspecific relaxations with a similar waveform are also produced by light of wavelengths >500 nm, which is not absorbed by Bis-Q. When the preparation was exposed to solutions containing agonist and Bis-Q, flashes were delivered alternately filtered through the blue and orange filters. During the off-line analysis of the digitized data, the latter episodes were substracted from the former. The substraction procedure was improved slightly if the nonspecific responses were multiplied by a scaling factor (between 1 and 2) before and after the flash. Presumably, the scaling factor depends on the detailed characteristics of the filters and the action spectrum for the nonspecific effect.

RESULTS

Bis-Q Binds to Muscarinic Receptors

Bis-Q inhibits the binding of [^{3}H]NMS to muscarinic receptors of frog myocardium (Fig. 1). The data fit closely to simple mass action curves, which suggests an interaction with a uniform population of binding sites. In the experiment of Fig. 1, the calculated dissociation constant of *trans*-Bis-Q is 4.3 ± 0.3 μM, whereas for the solution containing 80% *cis* isomer, the dissociation constant is 14.8 ± 1.4 μM. The latter value is considerably lower than that predicted (22 μM) if *cis*-Bis-Q is not bound or is bound with very low affinity. In a second experiment, the dissociation constants were 5.3 ± 0.5 μM for a

solution of *trans*-Bis-Q and 13 ± 4 μM for a sample of 90% *cis*-Bis-Q, again much less than predicted if *cis*-Bis-Q binds with negligibly low affinity. Therefore, *cis*-Bis-Q does bind to muscarinic receptors and it can be calculated from these results that the dissociation constant is 15–20 μM, or three- to fivefold weaker than the *trans* isomer.

The close fit of the binding curves to simple mass-action isotherms suggests that *cis*- and *trans*-Bis-Q interact competitively with [^{3}H]NMS. In further

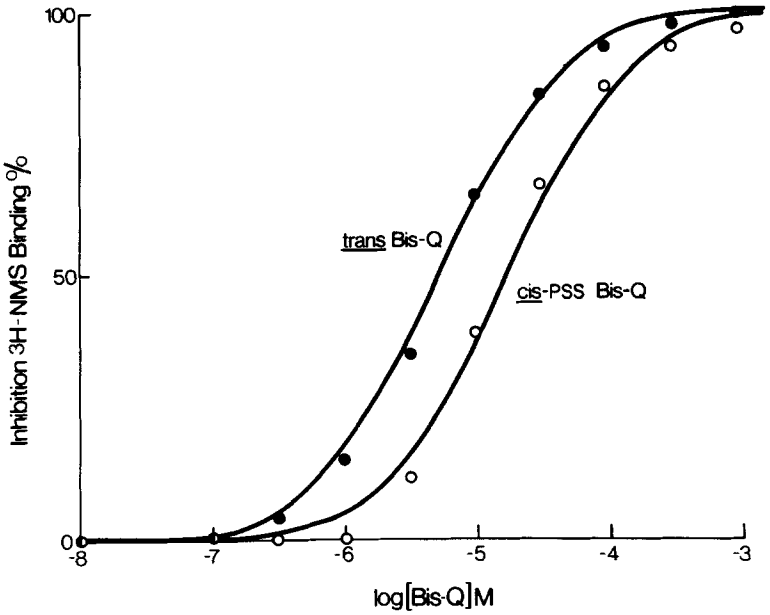

FIGURE 1. Inhibition of [^{3}H]*N*-methylscopolamine binding to frog myocardial muscarinic receptors by Bis-Q in the pure *trans* configuration (●) and in a mixture containing 80% *cis* configuration, close to the *cis*-photostationary state (○). Concentration of [^{3}H]NMS = 2.7×10^{-10} M. The receptor concentration was 54 pM. The curves are nonlinear least-squares best fits of the data to simple mass action curves with apparent log affinity constants (K_{app}) of 5.24 ± 0.02 (*trans*) and 4.7 ± 0.05 (80% *cis* solution). These values must be corrected by +0.13 log unit to give the log affinity constant (K) because of the relatively high receptor occupancy (35%) by [^{3}H]NMS. ($K = K_{app}$ (1 + K_{NMS} [NMS]); $K_{NMS} = 1.3 \times 10^9$ M^{-1}.)

experiments, a dose-ratio analysis showed that this interaction obeys the equation,

$$K = K_{app}(1 + K_{NMS}[NMS]) \quad (1)$$

where K is the affinity constant of Bis-Q, K_{app} is the apparent affinity constant measured at a given [^{3}H]NMS concentration, and K_{NMS} is the independently measured affinity constant of [^{3}H]NMS (1.3×10^9 M^{-1}). The apparent affinity constant K_{app} was measured for Bis-Q at two [^{3}H]NMS concentrations (2.7×10^{-10} M and 2.85×10^{-9} M), as in Fig. 1. For a competitive interaction, the

predicted ratio K_{app_1}/K_{app_2} is $(1 + K_{NMS}\,[NMS]_2)/(1 + K_{NMS}\,[NMS]_1)$, or log $(K_{app_1}/K_{app_2}) = 0.54$. The measured values were log $(K_{app_1}/K_{app_2}) = 0.48 \pm 0.08$ for *trans*-Bis-Q and 0.62 ± 0.09 for a solution of 90% *cis*-Bis-Q. Thus the inhibition curves for *cis*- and *trans*-Bis-Q are shifted to the right by [^{3}H]NMS in a parallel manner to the extent predicted for competitive interaction.

Bis-Q Inhibits the Conductance Induced by Muscarinic Agonists

Muscarinic agonists induce a potassium conductance in frog atrial fibers (Trautwein and Dudel, 1958; Hutter, 1961; Hartzell et al., 1977; Garnier et al., 1978*a*). In most of our experiments, we used carbachol (Carb) to evoke

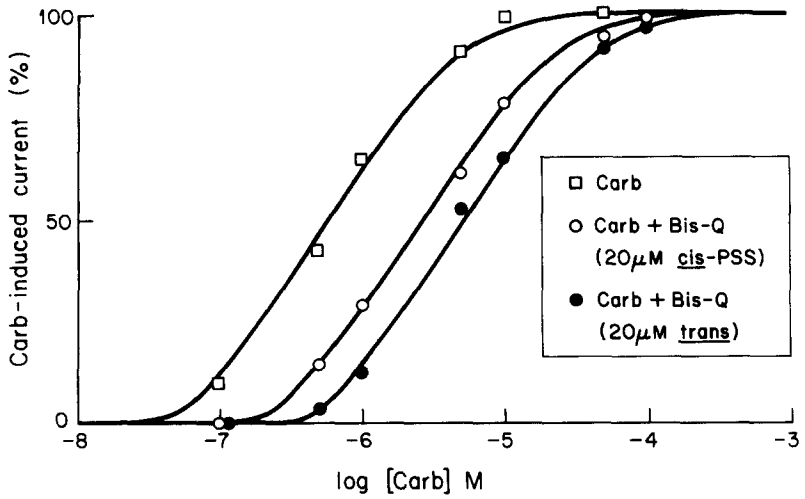

FIGURE 2. Inhibition of agonist-induced currents by Bis-Q. Responses are shown for Carb alone (□) and in the additional presence of 20 μM Bis-Q (●, pure *trans*; ○, 80% *cis*, close to the *cis*-photostationary state). 100% corresponds to 290 nA.

outward currents. The agonist-induced currents are stable and reproducible over time, showing no desensitization for the 5-min duration of a typical Carb application and a variation of <5% from one application to the next. The agonist-induced currents are inhibited by Bis-Q at concentrations in the range of 1–200 μM. The dose-response curve was shifted to the right by both the *cis* and *trans* configurations, and the *trans* configuration had a greater effect (Fig. 2). There was no change in the maximal response. A dose-ratio analysis (Arunlakshana and Schild, 1959) confirms that *trans*-Bis-Q is a competitive antagonist with an apparent dissociation constant of 5 μM (Fig. 3), in excellent agreement with the binding data.

The blockade by the 80% *cis* solution is too strong to be caused simply by the 20% *trans* configuration present. Furthermore, in an experiment with 20 μM pure *cis*-Bis-Q and 5 μM Carb, agonist-induced currents were still inhibited by 10–20%. The same solution caused a 60% inhibition when it was irradiated with visible light to form the *trans* photostationary state (65% *trans*).

Thus, *cis*-Bis-Q is a muscarinic blocking drug, albeit much weaker than the *trans* configuration. From experiments like that of Fig. 2, we estimate that the dissociation constants differ by a factor of three to five, also in excellent agreement with the binding data.

FURTHER OBSERVATIONS Several other photoisomerizable azobenzene

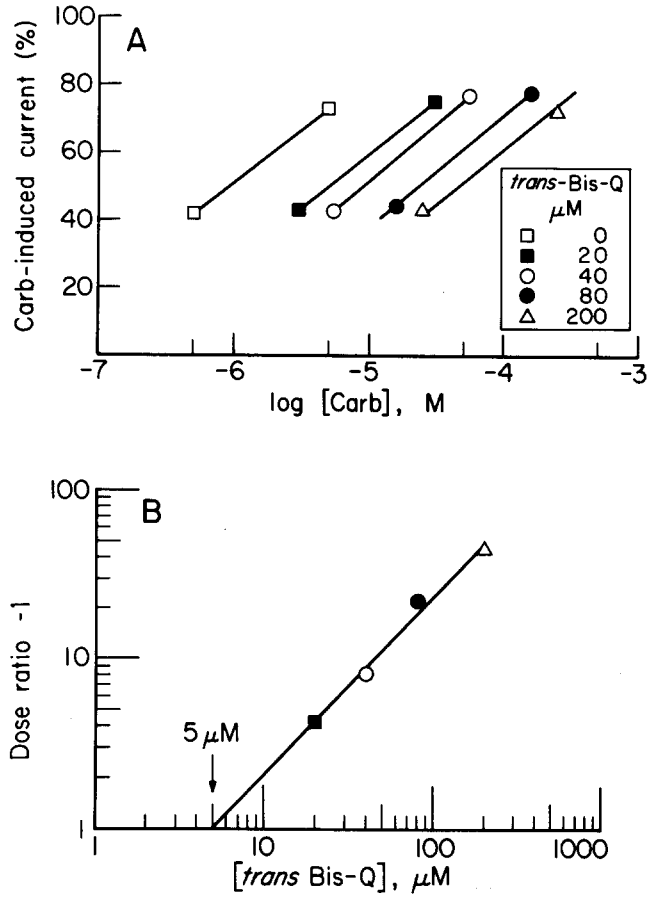

FIGURE 3. (A) Dose-response curves for carbachol in the presence of various concentrations of *trans*-Bis-Q. (B) Schild plot of the data in A (Arunlakshana and Schild, 1959). The dose ratio is the ratio between carbachol concentrations giving equal responses in the presence and absence of *trans*-Bis-Q. The least-squares line is drawn with a slope of 1.05 and an intercept of 5.0 μM.

compounds block the agonist-induced K^+ current in the same concentration range as Bis-Q. Furthermore, their *trans* configurations are the more potent blockers. Drugs showing such action include the 2,2′ and 4,4′ analogues of Bis-Q (Wassermann et al., 1979; Lester et al., 1980*b*), *n*-*p*-phenylazophenyl carbamylcholine (EW-1), and its *N*-methyl and *N*-phenyl derivatives (Bieth et al., 1970; Lester et al., 1979). Because we have access to large quantities of

Bis-Q and because we have substantial information on its photochemistry (Lester et al., 1980*a*), we used this compound in most of the experiments.

In frog atrial fibers, depolarization activates a slow inward current, presumably carried by Ca^{++} and Na^{+} ions. This current is blocked by low concentrations of muscarinic agonists (Giles and Noble, 1976; Garnier et al., 1978*b*; Nargeot et al., 1982) and in the course of the present experiments, we confirmed the observation that the current is enhanced by atropine, possibly because small levels of acetylcholine remain in dissected preparations (Giles and Noble, 1976). We also found that the slow inward current is enhanced by Bis-Q at micromolar concentrations (no systematic studies were performed to compare the stereoisomers).

Light-Flash Relaxations

As expected from the data described above, the membrane conductance could be modulated by light in preparations exposed simultaneously to agonist and Bis-Q. The agonist-induced conductance was *increased* by *trans* → *cis* photoisomerizations and *decreased* by *cis* → *trans* photoisomerizations.

The sucrose-gap technique gives the most stable recordings when the two sucrose pools and the central compartment are continually perfused with fresh solution. A few preparations, however, yielded good data even if the central compartment was not continually refreshed. With such preparations, we found that the flash-induced changes in the currents were maintained for at least several minutes (as long as we observed). Successive flashes produced cumulative effects that leveled off after several flashes, presumably as the *cis*/*trans* ratio reached photochemical equilibrium.

In most of our experiments, the central compartment was continually perfused with fresh solution (several chamber volumes per minute), and the conductance returned to its previous level with a time constant of 5–20 s (see traces in Figs. 4–6). Relaxations could therefore be repeated with good reproducibility at intervals of 40 s.

TIME COURSE The responses to flashes all have the same general features, independent of whether the relaxation constitutes a conductance increase or decrease (produced by net *trans* → *cis* or *cis* → *trans* photoisomerizations, respectively). The waveform is S-shaped. There is a delay, or period of zero slope, lasting several tens of milliseconds. The conductance change then accelerates, passes a point of inflection, and approaches the new steady state along a nearly exponential time course over the next few seconds.

These features are revealed most simply in conductance increases produced by *trans* → *cis* photoisomerizations using the 341-nm interference filter (Fig. 4). This filter eliminates the entire spectrum, except for a small amount of light near the absorption maximum of *trans*-Bis-Q. Therefore, we observe none of the nonspecific effects described in Methods. However, the low intensity (see Table I) does result in very small relaxations, never exceeding 5% of the agonist-induced current. Hence, it is necessary to average successive episodes in order to improve the signal-to-noise ratio.

Considerably larger relaxations—up to 20% of the agonist-induced cur-

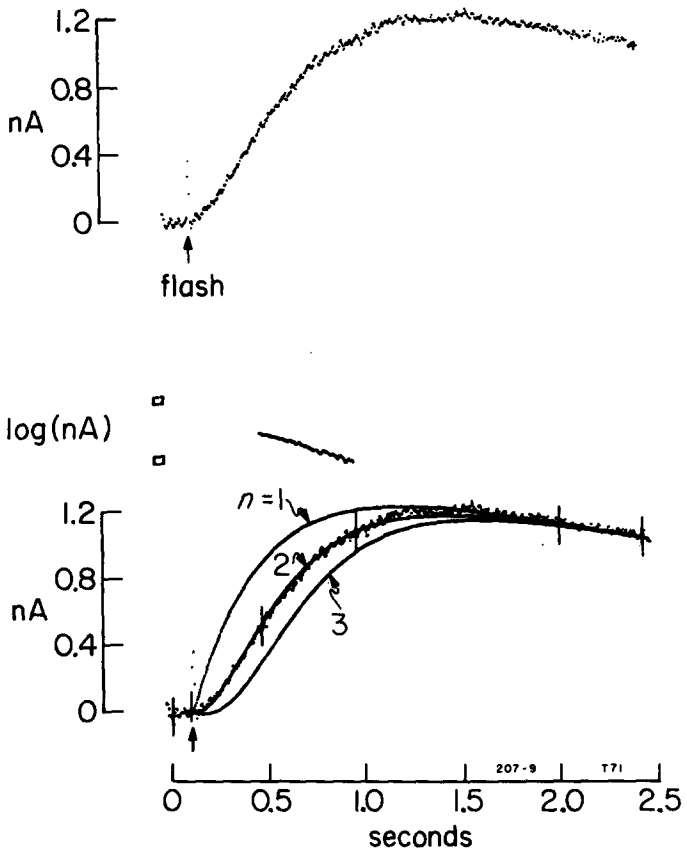

FIGURE 4. Relaxations after *trans* → *cis* photoisomerizations. The upper panel shows voltage-clamp current in a preparation exposed to 20 μM *trans*-Bis-Q and 5 μM Carb and clamped at +20 mV with respect to the resting potential in the absence of agonist. The agonist-induced current is 32 nA. The standard flash is used with the 341-nm filter (see Methods). The trace is the average of four episodes, taken at intervals of 40 s and passed through a recursive digital filter with a time constant of 5 ms. The lower panel shows how the data are described by the parameters of Eq. 1. Three pairs of cursors are superimposed on the waveform. The leftmost pair delimits the portion used to calculate the current just before the flash. The portion between the rightmost pair is used to calculate a linear trend. This line, presumably due to perfusion with unisomerized solution, is extrapolated back to the time of the flash (not shown). The baseline is subtracted from this value to give the relaxation amplitude, ΔI_o = 1.5 nA. The rate constant k is estimated from the portion between the middle pair of cursors (beginning roughly at the inflection point). The approach to the sloping equilibrium line is nearly exponential; the slope is 2.5 s^{-1} on semilogarithmic coordinates (boxes define a 10-fold range). This value provides the initial estimate of k for an interactive routine that fits Eq. 1 to the data. The smooth line superimposed on the data is a good fit with k = 2.8 s^{-1}, n = 2. For comparison, lines are also shown with n = 1 and 3.

rent—are observed if the standard flash is used in conjunction with more transparent filters, although with such bright flashes it is necessary to substract the nonspecific relaxations observed in the absence of photosensitive drugs (see Methods). Fig. 5 shows increases in response to *trans* → *cis* photoisomerizations produced with the "UV only" filter, and Fig. 6 shows the conductance decrease that results when visible flashes are delivered to a preparation bathed in agonist and *cis*-Bis-Q. The relaxation waveforms have the same features as those observed with the 341-nm filter: an initial delay, an accelerating change, and a nearly exponential approach to a new steady state.

The nonspecific effects constituted a minor inconvenience in most of our experiments. Subtracting them changed only one feature of the response to Bis-Q: the rapid jump just after the flash was eliminated. In particular, we emphasize that the subtraction procedure does not substantially alter the period of zero slope (or delay) just after the flash, because both the specific and nonspecific traces are nearly flat during this period (Figs. 5 and 6).

TABLE I

Filter	Transmission	Use	K_c	K_t
			1/flash	
UG11, UV only	λ_{max} = 320 nm	*trans* → *cis*	0.81	1.04
341 nm interference	341 nm	*trans* → *cis* (no nonspecific effects)	0.002	0.06
BG3, blue	λ_{max} = 370 nm	*cis* → *trans*	0.63	0.22
OG530, orange	transmits > 500 nm	flash without affecting Bis-Q	<0.01	<0.01

Photoisomerization potencies for the standard flash, with the filters used in the experiments. Broadband filters are described both by the light they transmit and by the catalog listing of the manufacturer (Schott Optical Glass Inc., Duryea, Pa.). The interference filter was supplied by Balzers, Hudson, N. H.

MATHEMATICAL DESCRIPTION In describing the effect of experimental manipulations, it is convenient to abstract the relaxation waveform to a small number of parameters. The light-flash relaxations are fit quite well by an expression of the form

$$\Delta I = \Delta I_0(1\text{-}e^{-kt})^n, \quad (2)$$

where ΔI is the relaxation amplitude (either positive or negative) and t is the time since the flash. Our most complete data were taken at a carbachol concentration of 5 μM, a Bis-Q concentration of 20 μM, and a temperature of 22–24°C. For 19 preparations tested under these conditions, k was 3.23 ± 0.54 s^{-1} (mean ± SD, range 2.4–4.1). If the relaxation were a simple exponential approach to a new steady state, the parameter n would equal unity. Larger values are associated with longer initial delay periods. For the conditions given, n was usually 2 (2.1 ± 0.24, range 1.8–2.5).

Only Temperature Strongly Affects Relaxation Waveforms

We tested several experimental manipulations for their effects on light-flash relaxations. Responses were slower in cooled preparations, and faster in warmed preparations. The data of Table II show that k has a Q_{10} between 2

and 2.5. There were small increases in n for preparations cooled below room temperature, but these have doubtful significance because the agonist-induced conductances also decreased by about threefold, decreasing the accuracy of the measurements.

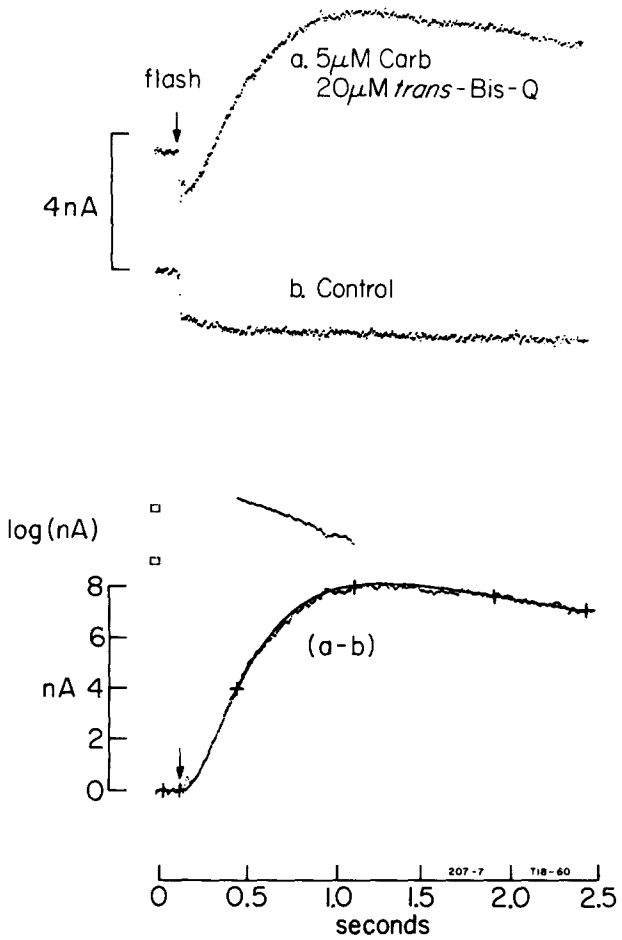

FIGURE 5. Relaxations after *trans* → *cis* photoisomerizations with the "UV only" filter. Other conditions are identical to those for the experiment of Fig. 3. The two traces in the upper panel are single episodes taken (*a*) in Carb and *trans*-Bis-Q and (*b*) in Ringer solution. Agonist-induced current is 53 nA. In the lower panel, the two traces have been subtracted and filtered with a time constant of 10 ms. The semilog plot has a slope of 3.1 s^{-1}; the smooth curve is generated by Eq. 1 with $k = 3.4\ s^{-1}$, $n = 2$.

RELAXATIONS AT DIFFERENT VOLTAGE Membrane voltage had little or no effect on the kinetics of the relaxations (Table III). Measurable relaxations were obtained at voltages between 0 mV (i.e., the resting potential in the absence of agonist) and +80 mV. Over this range, the rate constant k changed by <20%. Because individual measurements have an uncertainty of at least

10%, this small trend may not reflect a real property of the system. If the data of Table III are analyzed assuming that k depends exponentially on voltage, a least-squares fit reveals a rather weak dependence of e-fold for ~400 mV.

Measurements become less precise at voltages more depolarized than +80

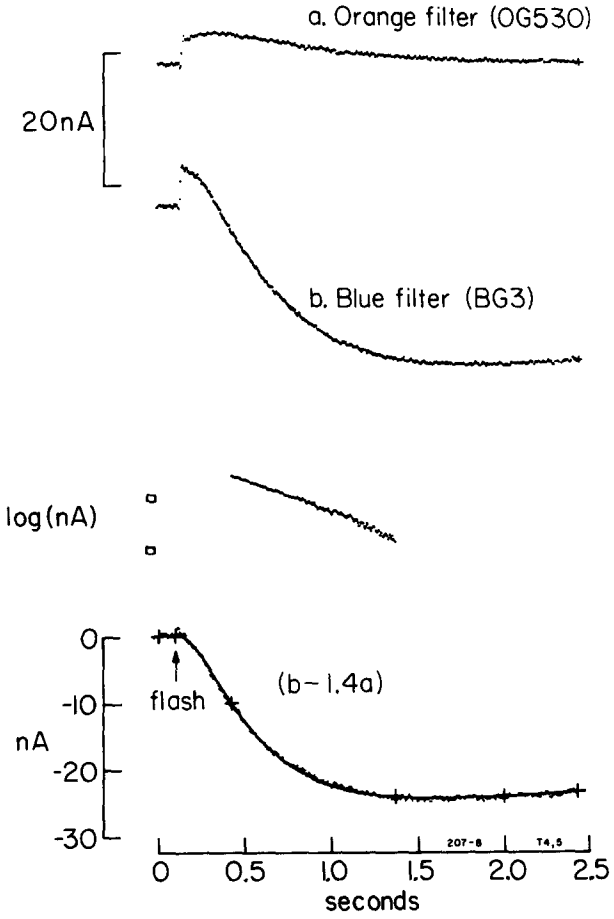

FIGURE 6. Relaxations after *cis* → *trans* photoisomerizations. In the upper panel, both episodes are taken with the preparation exposed to 5 μM Carb and to 20 μM Bis-Q (80% *cis* configuration, close to the *cis* photostationary state). The agonist-induced current is 220 nA. The lower panel shows subtracted and filtered data (see Methods). The semilog plot has a slope of 3.0 s^{-1} and the smooth line is a fit to Eq. 1 with k = 3.2 s^{-1}, n = 2. Other details as in the experiments of Figs. 3 and 4.

mV because the agonist-induced currents are superimposed upon large delayed rectifier currents. Nonetheless, we observed no major changes in the light-flash relaxations at +100 mV.

Agonist-induced currents are small and inward at voltages more negative than about −20 mV with respect to the normal resting potential. At −40 mV,

we did observe relaxations with inverted sign. However, the signals were too small for accurate analysis.

RELAXATIONS WITH DIFFERENT AGONISTS In one experiment, we compared light-flash relaxations with Carb, acetylcholine, and acetyl-β-methylcholine, all at a concentration of 5 μM. With the procedure of Fig. 4, we found that the data were fitted well by $k = 3.1$–$3.2\ s^{-1}$, $n = 1.8$ in each case. In another experiment, we compared Carb and muscarine, and again found that the waveforms had identical shapes. Thus, the light-flash relaxations do not depend on the nature of the agonist.

TABLE II

Temperature	k, s^{-1}	n	Number of preparations
		mean ± SD	
13–16°C	1.6	2.65±0.7	6
23–24°C	3.4	2.14±0.23	7
29–30°C	5.6	2–2.5	2

Effect of temperature on the kinetics of light-flash relaxations. Data are from seven different preparations, each tested at two or more temperatures. The agonist was 5 μM Carb with Bis-Q present at 20 μM, *cis*-PSS. The standard flash was used with the blue filter, as in the experiment of Fig. 4. Values of k and n are given for best fits to Eq. 1 (see Fig. 3).

TABLE III

Membrane potential	k, s^{-1}
mV	*mean ± SD*
0	2.97±0.40
+20	2.84±0.54
+40	3.10±0.17
+60	3.37±0.31
+80	3.50±0.30

Light-flash relaxations at various membrane potentials. Data are averaged from three different preparations, each tested at all indicated voltages. The trials were conducted at 22–24°C, with Bis-Q present at 20 μM, *cis*-PSS. The standard flash was used with the blue filter as in the experiment of Fig. 4. Values of k are given for best fits to Eq. 1 (see Fig. 3). In each case, $n = 2$ gave the best fit.

RELAXATIONS AT VARIOUS CARBACHOL CONCENTRATIONS Our kinetic data are less precise at agonist concentrations much greater or less than the midpoint of the dose-response curve. At low agonist concentrations, the agonist-induced conductances are small. At high concentrations, the agonist-induced conductances are large but the relaxations are small, presumably because measurements are carried out near the plateau of the dose-response curves (see Fig. 2). Furthermore, at high agonist concentrations, the nonspecific relaxation for visible flashes was quite large. These factors would vitiate any attempt to study small effects of agonist concentration. It is clear, however, that there were no large changes (Table IV). The rate constant k did increase

slightly with agonist concentration, but the change amounted to a factor of less than two for a 50-fold range of Carb concentration that effectively covers the entire dose-response relation (see Fig. 2). In view of the possibility of systematic errors arising from non-isopotentiality and other artifacts, we consider it doubtful that the small observed trend reflects a real property of the response to agonist.

RELAXATIONS AT VARIOUS BIS-Q CONCENTRATIONS In three different preparations, we failed to detect an effect of changes in Bis-Q concentration on the shape of the relaxation waveform; Fig. 7 presents data from one such experiment. For reasons similar to those just given, the relaxations are too small for reliable measurements outside a narrow range of Bis-Q concentrations. In terms of Eq. 1, the relaxations of Fig. 7 were characterized by k in the range of 2.8–3.5 s^{-1} and by $n = 1.8$.

TABLE IV

[Carb]	k, s^{-1}	Number of preparations
μM	*mean ± SD*	
1	2.58±0.17	3
2	3.21±0.25	3
5	3.30±0.47	5
50	4.39±0.89	4

Light-flash relaxations at various Carb concentrations. Each preparation was exposed to Carb at 5 μM and at least one other concentration. The procedure was that of Fig. 3 (*trans* → *cis* photoisomerization) or Fig. 4 (*cis* → *trans*), and other conditions were identical to those of Figs. 3 and 4. The best value for n was between 2 and 2.5 in all cases.

DISCUSSION

In this study we show that Bis-Q binds to muscarinic receptors and that its action is well described as competitive antagonism. We use this photoisomerizable molecule to study kinetics of the muscarinic responses. At present the technique can produce only fractionally small (< 20%) changes in the agonist-induced currents, both because the *cis* and *trans* configurations do not differ greatly in their pharmacological properties, and because flashes can shift the isomeric composition toward equilibrium mixtures rather than toward the pure *cis* or *trans* isomers (see Lester et al., 1980*a*). Nonetheless, it is already possible to conclude that the conductance transients—both increases and decreases—have S-shaped waveforms and that the time course is affected only by temperature and not by several other experimental manipulations.

Is Bis-Q a Competitive Antagonist?

The binding data alone cannot exclude the possibility that [^{3}H]NMS and *cis*- or *trans*-Bis-Q may be interacting in a heterotropic cooperative manner with a negative cooperativity of >100; such interactions are difficult to distinguish from simple competitive behavior. Similarly, the dose-response data alone cannot exclude the possibility that Bis-Q is a noncompetitive antagonist and

that the preparation contains a 50-fold excess of "spare receptors". Taken together, however, the biochemical and electrophysiological data provide consistent and satisfying evidence for purely competitive antagonism under the conditions of the light-flash experiments.

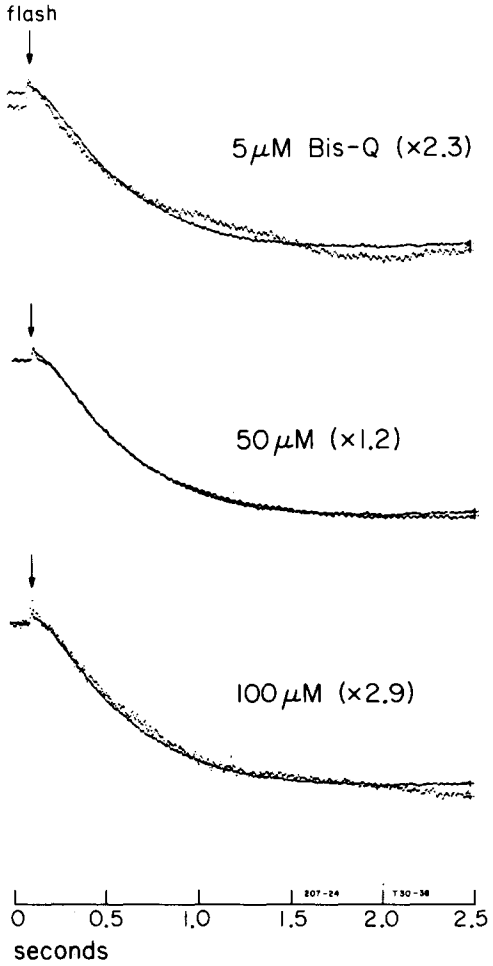

FIGURE 7. Light-flash relaxations in various concentrations of Bis-Q. Conditions were the same as in the experiment of Fig. 6. The smooth trace which appears in all panels is the relaxation in 20 μM Bis-Q (average of four episodes). The light-flash relaxation has an amplitude of 7.8 nA. This trace is superimposed on appropriately multiplied traces from trials at other concentrations (average of two or three episodes). Arrows give times of flash.

Comparison with Previous Studies

In experiments with micro-iontophoretic application of agonists, voltage transients are proportional to the third power of time (Hartzell et al., 1977; Pott, 1979; Hill-Smith and Purves, 1978) rather than the square as seen here. There are at least two possible explanations for this apparent discrepancy.

First, in our light-flash experiments, the relaxations might follow a simpler time course either because they take against a background of partially liganded receptors or because they represent fractionally small perturbations of the conductance. Second, receptor kinetics might have been partially distorted in earlier studies either by the membrane time constant or by spread of transmitter (Hartzell, 1980; Osterrieder et al., 1980). In the present experiments these two complications are avoided by the combined use of the voltage clamp and the concentration-jump technique.

Noma and Trautwein and their collaborators have reported a series of experiments on the muscarinic potassium conductance of rabbit sinoatrial node (Noma and Trautwein, 1978; Noma et al., 1979*a* and *b*; DiFrancesco et al., 1980; Osterrieder et al., 1980). Their results suggest that there is a potassium channel gated by a single bound agonist molecule, that the opening rate increases with depolarization, and that these transitions are governed by a single rate-limiting step. Our data are less complete but it is already possible to make some comparisons with the rabbit sinoatrial node. The most important similarly is the time scale of the kinetics. The average rate constants (angular corner frequencies) reported by Noma et al. (1979*a*) for acetylcholine (10^{-5} M) were 5.9 s^{-1} at 37°C and 2.1 s^{-1} at 27°C, only a factor of two less than the values expected for the same temperatures from the data of Table II. DiFrancesco et al. (1980) also showed that potassium accumulation did not distort their measured kinetics; such artifacts are also unlikely in the present study because voltage jumps are not involved.

There are also several important differences. First, Noma and Trautwein (1978) reported single exponential time courses for voltage-jump relaxations and Noma et al. (1979*a*) reported single Lorentzians for fluctuation power spectra; but we find that light-flash relaxations begin with a delay and are best described as the *square* of a single exponential ($n = 2$ in Eq. 1). This difference, like the others we shall note, could arise simply from the use of different preparations. On the other hand, it seems possible that the flash-induced concentration jumps perturb the system at an earlier step than the one that is affected by voltage jumps or the one that dominates spontaneous fluctuations. If, for instance, the delay is introduced after the transmitter-receptor interaction and before the voltage-sensitive step, this would explain the different observed waveforms. A technical point also deserves consideration: in a voltage-jump relaxation an initial, nonexponential component would overlap at least partially with the capacitive transient and might thus escape notice. In fluctuation experiments, our relaxations would probably correspond to a superposition of two Lorentzians with corner frequencies differing by only twofold; this might also escape detection.

Voltage sensitivity is another difference. We find that membrane potential has little or no effect on the waveform of the light-flash relaxations. We can give an upper limit on voltage sensitivity by assuming that only the channel closing rate, α, is sensitive to voltage (Sheridan and Lester, 1977; Sakmann and Adams, 1979; Osterrieder et al., 1980). Our data were taken at roughly the midpoint of the dose-response curve, so that the opening rate β' roughly

equals α. The total relaxation rate constant k, which equals $\alpha + \beta'$, would be roughly half as sensitive as α itself. The data of Table III allow the conclusion that the upper limit on the voltage sensitivity is e-fold per 400 mV; therefore, α is no more sensitive to voltage than e-fold per 200 mV. Thus, our study suggests that α is less than half as voltage sensitive as concluded by Osterrieder et al. (1980), if at all.

We emphasize our complete agreement that the muscarinic response *is voltage sensitive*: it decreases with depolarization (Garnier et al., 1978*a*; Noma and Trautwein, 1978). Therefore, some structure does change with membrane voltage. The point of discussion here is the rate of these changes. Our results suggest that they are much greater than the $\sim 3\ s^{-1}$ that characterizes our relaxations at 22–24°C.

A final difference concerns the effect of agonist concentration on the kinetics. We found no important effects over a 50-fold range of agonist concentration that effectively spans the dose-response curve. However, technical factors might well prevent our technique from resolving a twofold change in time constants over this range, as reported by Osterrieder et al. (1980).

Implications for the Rate-limiting Step

In experiments on nicotinic synapses, relaxation kinetics depend on the nature and concentration of the agonist. These facts suggest that conductance changes are limited in rate either by the binding and dissociation of the agonist or by an intramolecular event, such as a conformational change of the receptor protein, that is closely coupled to the binding event (Sheridan and Lester, 1975, 1977; Sakmann and Adams, 1979). The muscarinic receptor is clearly not characterized by such a close relation between binding and the activation of the conductance. We find no detectable influence of the nature of the agonist and a very weak, possibly insignificant, effect of concentration. The rates themselves are several orders of magnitude slower than the 10^2–$10^4\ s^{-1}$ one would expect if drugs at a concentration of 10^{-5} M are binding with rate constants of 10^7–$10^9\ M^{-1} \cdot s^{-1}$.

It would of course be preferable to conduct studies with a light-activated agonist rather than by relying on the less direct method of photoisomerizing a blocking molecule. Nonetheless, we find no effect of Bis-Q concentration. Therefore it also seems unlikely that the responses are governed by the molecular binding rates of the interaction between Bis-Q and the receptor. The present experiments are analogous to those conducted with the photoisomerizable competitive nicotinic antagonist 2BQ (Lester et al., 1980*b*). A comparison between these two studies again reveals the very different time course of nicotinic and muscarinic responses: the light-flash relaxations occur in a few tens of milliseconds at nicotinic receptors, but in a few seconds at muscarinic receptors.

Relaxations this slow can, however, be produced when free diffusion is slowed by repeated binding of drugs to receptors (Armstrong and Lester, 1979). Such a mechanism is rendered unlikely by the observation that kinetics are described by rate constants with a Q_{10} of 2–2.5 (Hartzell et al., 1977;

Hartzell, 1980; Pott, 1979), as confirmed in the present study. Furthermore, Armstrong and Lester (1979) showed that each of three conditions must be fulfilled if buffered diffusion can yield kinetics on a time scale of seconds: (*a*) agonist-receptor binding affinities must exceed $10^6\ M^{-1}$; (*b*) receptor densities must be on the order of $10^4/\mu m^2$; (*c*) receptors must be located in restricted extracellular clefts, on the order of 50 nm in width. None of these conditions are fulfilled at the muscarinic synapses of frog heart (cf. Hartzell, 1980).

The details of the relaxations provide further constraints on the nature of the rate-limiting steps. The relaxations are S-shaped under all experimental conditions and even for perturbations amounting to <5% of the agonist-induced conductance. Many membrane conductances show an S-shaped time course under specific experimental conditions, usually activation from a zero start. For instance, such behavior is displayed by the electrically excitable sodium and potassium channels that underly the nerve impulse. The usual explanation is that the active state of the channel requires either the combined activation of several subunits (Hodgkin and Huxley, 1952) or the sequential completion of several steps (see for instance Armstrong, 1975) and that the microscopic transitions have voltage-dependent rate constants. However, the electrically excitable channels display nearly monoexponential kinetics under several other conditions, such as repolarizations that shut the channels, and for small perturbations.

The present data suggest, instead, a scheme that incorporates a rapid ligand-receptor interaction followed by several sequential slower steps of approximately equal speed.

$$\begin{array}{ccccccc} & & \text{antagonist}\cdot\text{receptor} & & & & \\ & & \uparrow\downarrow\ \text{fast} & & & & \\ \text{(agonist or antagonist)} & + & \text{receptor} & \overset{\text{fast}}{\longleftrightarrow} & \text{agonist}\cdot\text{receptor} & \overset{\text{slow}}{\longleftrightarrow} & \text{intermediate} \overset{\text{slow}}{\longleftrightarrow} \text{activated conductance} \end{array} \quad (3)$$

We have written scheme 3 in a rather general fashion. It includes the possibilities that more than one bound agonist molecule is required for activation; that the subsequent steps involve molecules distinct from the receptor itself (as discussed below); and that the rate-limiting steps involve bidirectional fluxes. The scheme does provide for a delay between any perturbation of the ligand-receptor complex and the conductance transient.

A specific hypothesis for the nature of the slow steps is that they involve transitions in a protein with two identical subunits. Although our evidence suggests that these transitions do not involve the actual binding of the transmitter molecule, there could be other ligand-protein reactions at a later step in the signal pathway. Alternatively, there could be conformational

changes in a protein. In either case, the transitions would conform to the scheme

$$P_2 \underset{k_-}{\overset{2k_+}{\rightleftharpoons}} P'P \underset{2k_-}{\overset{k_+}{\rightleftharpoons}} P'_2. \tag{4}$$

The statistical factors of 2 arise from the assumption that the subunits have equal and independent transition rates. The concentration of P_2 would change rapidly in response to a perturbation of the agonist-receptor interaction; these changes would be propagated to P'_2 after a delay; and the P'_2 state would rapidly affect the conductance. For this model, the active state P'_2 undergoes relaxations of the form

$$\Delta[P'_2] = \Delta[P'_2]_0[1 - \exp[-(k_+ + k_-)]^2 t, \tag{5}$$

which agrees with our data if $k = k_+ + k_-$.

Our studies shed little light on the specific identity of the postulated protein P involved in the rate-limiting kinetic steps. Because the kinetics have a Q_{10} of 2–2.5, we believe that these steps have activation energies of 13–17 kcal/mol. Because they are not sensitive to membrane potential, we have no evidence that they occur within the membrane. Several studies have yielded evidence that muscarinic responses involve the binding or action of intracellular second messengers. Possible candidates include cyclic AMP (Murad et al., 1962; George et al., 1973; Watanabe and Besch, 1978; Jacobs et al., 1979; Hulme et al., 1981), guanosine triphosphate (Berrie et al., 1979; Wei and Sulakhe, 1979; Rosenberger et al., 1979), phospholipids (Michell et al., 1975), and Ca^{++} ions (Putney et al., 1980; Salmon and Honeyman, 1980). In the context of scheme 3, it should be noted that cyclic AMP-sensitive protein kinase contains two identical regulatory subunits and two identical catalytic subunits and that activation requires the dissociation of the regulatory subunits.

It remains possible that in bovine conduction tissue the muscarinic receptors may be linked directly to K channels, as suggested by Trautwein and collaborators. In accord with this hypothesis, potassium ions appear to increase agonist binding, an effect seen only weakly with other ions (Burgen et al., in press). Thus the inactive state P_2 (scheme 3) would be the agonist-receptor complex itself, and the state P'_2 would be the activated channel itself.

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